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Separate Functional Domains of the Herpes Simplex Virus Type 1 Protease: Evidence for Cleavage inside Capsids

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The herpes simplex virus type 1 (HSV-1) protease (Pra) and related proteins are involved in the assembly of viral capsids and virion maturation. Pra is a serine protease, and the active-site residue has been mapped to amino acid (aa) 129 (Ser). This 635-aa protease, encoded by the UL26 gene, is autoproteolytically processed at two sites, the release (R) site between amino acid residues 247 and 248 and the maturation (M) site between residues 610 and 611. When the protease cleaves itself at both sites, it releases Nb, the catalytic domain (N_0), and the C-terminal 25 aa. ICP35, a substrate of the HSV-1 protease, is the product of the UL26.5 gene. As it is translated from a Met codon within the UL26 gene, ICP35 c,d are identical to the C-terminal 329-aa sequence of the protease and are *trans* cleaved at an identical C-terminal site to generate ICP35 e,f and a 25-aa peptide. Only fully processed Pra (N_0 and Nb) and ICP35 (ICP35 e,f) are present in B capsids, which are believed to be precursors of mature virions. Using an R-site mutant *A247S* virus, we have recently shown that this mutant protease retains enzymatic activity but fails to support viral growth, suggesting that the release of N_0 is required for viral replication. Here we report that another mutant protease, with an amino acid substitution (Ser to Cys) at the active site, can complement the *A247S* mutant but not a protease deletion mutant. Cell lines expressing the active-site mutant protease were isolated and shown to complement the *A247S* mutant at the levels of capsid assembly, DNA packaging, and viral growth. Therefore, the complementation between the R-site mutant and the active-site mutant reconstituted wild-type Pra function. One feature of this intragenic complementation is that following sedimentation of infected-cell lysates on sucrose gradients, both N-terminally unprocessed and processed proteases were isolated from the fractions where normal B capsids sediment, suggesting that proteolytic processing occurs inside capsids. Our results demonstrate that the HSV-1 protease has distinct functional domains and some of these functions can complement in *trans*.

Herpesvirus capsids are icosahedral protein shells approximately 15 nm in thickness and 125 nm in diameter. Three types of capsids, termed A, B, and C, can be isolated by sucrose gradient centrifugation (15). C capsids contain the entire viral genome and are able to mature into infectious virus. A and B capsids lack viral DNA and are found in the infected-cell nucleus. A capsids are thought to result from failed attempts at packaging viral DNA, while B capsids are thought to be intermediates in capsid assembly, because they differ from A and C capsids by the presence of large amounts of the viral assembly protein ICP35 (VP22a) (3, 15, 35, 36, 41). There is a family of ICP35 proteins designated ICP35 a through f (3). Although all forms of ICP35 can be detected on Western blots (immunoblots) of infected cells, only forms ICP35 e,f are present in B capsids (1, 7, 15, 35, 41, 46). Because ICP35 e,f are present in B capsids and absent from the mature virions, it has been suggested that the role of ICP35 e,f in capsid assembly is analogous to that of the scaffold proteins of double-stranded bacteriophage (4, 14, 16).

In addition to ICP35 e,f, B capsids are composed of at least seven other proteins: VP5, VP19C, Nb (VP21), VP23, N_0 (VP24), VP26, and the recently reported UL6 gene product (1, 16, 37, 40, 43, 52). Three capsid proteins are encoded by a single open reading frame (ORF) designated UL26, and two transcripts have been mapped to this ORF (20, 25, 33). One transcript, designated UL26, encodes the 635-amino-acid (aa) protease. The other more abundant shorter transcript, designated UL26.5, encodes the 329-aa protein ICP35 (26, 45). Since residue 307 (Met) of Pra is also the start codon for ICP35, the two transcripts are in frame and are 3′ coterminal (19, 25, 26).

The full-length herpes simplex virus type 1 (HSV-1) protease (Pra) undergoes autoproteolytic processing at two sites, the release (R) site and the maturation (M) site, which lie between Ala and Ser at residues 247 and 248 and residues 610 and 611, respectively (9, 42). Cleavages at these sites generate N_0, Nb, and a 25-aa peptide (Fig. 1A). Identification of N_0 (the N-terminal 247 aa of Pra) as the catalytic domain of the protease suggests that the HSV-1 protease has multiple domains (27, 28, 53). Since ICP35 overlaps and is in frame with the C-terminal half of Pra, ICP35 c,d can be *trans* cleaved at its C terminus by the protease to generate ICP35 e,f and a 25-aa peptide (Fig. 1A).

Another herpesvirus protease, the product of the cytomegalovirus (CMV) UL80 gene, in addition to autoproteolysis at R and M sites (56), has a third internal (I) cleavage site located in the middle of the catalytic domain (2, 21, 39, 54). The CMV protease can *trans* cleave the HSV-1 substrates, but the HSV-1 protease cannot cleave the CMV substrates (55).

The HSV-1 protease is a serine protease, and its active site has been mapped to Ser-129 (11, 27). Its requirement for

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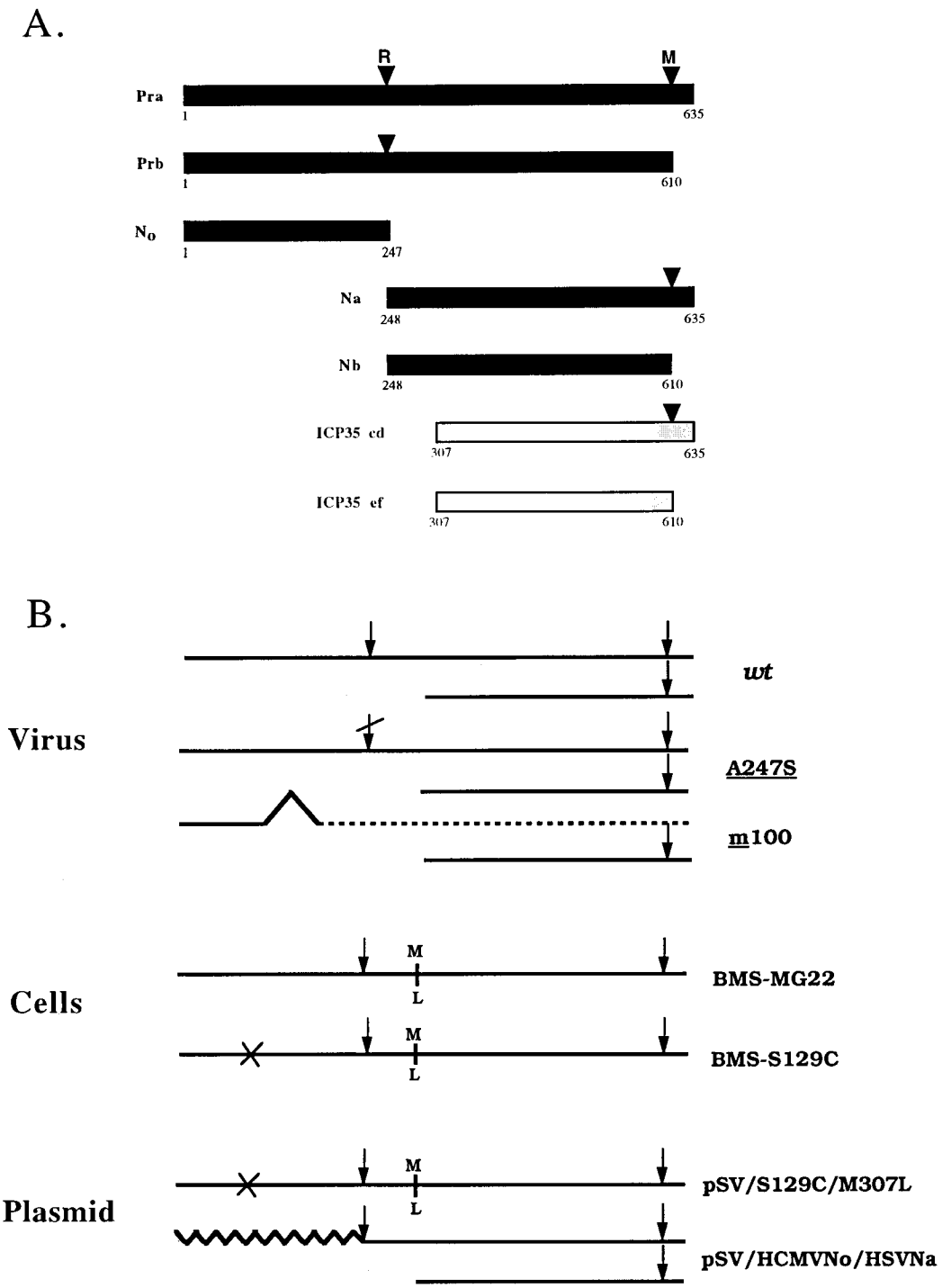


FIG. 1. (A) Polypeptide products of UL26 and UL26.5 ORFs. The HSV-1 protease (Pra), substrate (ICP35 c,d), and cleavage products Prb, N_0 (VP24), Na, Nb (VP21), and ICP35 e,f (VP22a) are described in the text. The cleavage sites (R and M sites) of Pra and ICP35 c,d are indicated by arrowheads. The UL26 amino acid numbers of the N and C termini of each protein are indicated. (B) The ORFs of HSV-1 wt and mutant protease plasmids, cell lines, and viruses. Arrows indicate cleavage sites. A slash through an arrow denotes an uncleavable site due to a point mutation at P1. The dashed line indicates a frameshift in the ORF of the catalytic domain of the protease, rendering it inactive, while leaving the ICP35 ORF unaffected. An X through the protease ORF indicates a point mutation at the active-site serine, rendering it inactive. M over L indicates that the methionine at 307 (start codon for ICP35) is changed to leucine. The wavy line denotes the HCMV protease. Construction of these mutants is described in Materials and Methods.

proper capsid assembly confirmed that the protease is essential for the production of infectious virus (13, 44). Although capsid structures are observed in the absence of functional protease, the major capsid protein VP5 does not adopt the correct conformation and viral DNA is not encapsidated (44). Genetic and functional analyses revealed that the full-length protease (Pra), the catalytic domain (N_0), and the R and M cleavage site mutant proteases are proteolytically active (26, 32, 53). How-

ever, the catalytic domain of the protease, N_0, alone is insufficient to support viral growth, suggesting that Na may be required to direct the catalytic domain to the site of capsid assembly (13). These results also indicate that in addition to enzymatic activity, the HSV-1 protease may have additional functions required for viral replication.

Three products of proteolytic processing, N_0, Nb, and ICP35 e,f, are found in B capsids (15, 37, 52, 53), but the fate of the fourth product, the C-terminal 25-aa peptide of both Pra and ICP35 c,d, is unknown. We and others recently reported that the C-terminal 25-aa peptide of Pra and ICP35 are involved in the formation of sealed capsids and may directly interact with VP5 (8, 13, 22, 31, 38, 50).

In this study, we report that two lethal mutant proteases can complement each other in *trans*. This intragenic complementation was observed at the levels of capsid assembly, DNA packaging, and viral growth. Our results demonstrate that the HSV-1 protease is a multifunctional protein and some of its functional domains can be genetically separated. One feature of the complementation between these two mutants is that following sedimentation of infected-cell lysates, Prb was isolated from the fractions where normal B capsids sediment. Thus, the precursor of N_0 and Nb can be incorporated into capsids, suggesting that proteolytic processing occurs inside capsids.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (23). The growth medium for the neomycin-resistant cell lines BMS-MG22 and BMS-S129C (see below) included 250 μg of the antibiotic G418 per ml. BMS-MG22 cells (Fig. 1B) express full-length HSV-1 protease with a Met-to-Leu change at residue 307, which can fully support the growth of protease mutant viruses (13).

The wild-type (wt) HSV-1 strain KOS1.1 was propagated and assayed as described previously (23, 24). The mutant protease viruses *m*100 and *A247S* were grown in BMS-MG22 cells.

Plasmids. Construction of plasmids pM307L, pSVPra, $pSVN_0$, and pUCICP35 has been described previously (13, 29, 31). Plasmid $pSV/HCMVN_0/HSVNa$ was constructed as follows: a 492-bp *Pst*I-*Apo*I fragment containing the human CMV (HCMV) catalytic domain gene and the R-site junction of HCMV and HSV-1 protease gene was generated by standard PCR technique by using pT7CMVProA as a template (12) and two oligonucleotides (5′-GGGTCTTT TTTGCCTGGGCTGCGTCACTTCGCCAGG-3′ and 5′-AAGGCC**GAATTTT TCGCTCGCCT**TGACG-3′) as primers. The sequences which are underlined and in bold type represent the coding region at the R site of the HCMV catalytic domain and HSV-1 Na, respectively. Plasmid $pHCMVN_0/HSVNa$ was constructed by three-way ligation of the PCR product, a 3,438-bp *Pst*I-*Asp*718I fragment of $pT7HCMVN_0C$ and a 1,180-bp *Apo*I-*Asp*718I fragment of pT7635A. Plasmid $pSV/HCMVN_0/HSVNa$ was constructed by ligation of the *Xba*I-*Asp*718I fragment of $pHCMVN_0/HSVNa$ into pJ3Ω (34). The coding region at the junction between $HCMVN_0$ and HSVNa was confirmed by DNA sequencing. Plasmid $pSV/HCMVN_0$ was constructed by ligation of the *Xba*I-*Asp*I fragment of $pT7HCMVN_0C$ into pJ3Ω. Plasmid $pGSTN_0S129C$ was constructed by standard PCR techniques, and the mutation was confirmed by elimination of the *Msc*I site, with the generation of *Bpu*11021 site. Plasmid pSV/S129C/M307L was constructed by three-way ligation of the 364-bp *Eag*I-*Hpa*I fragment of $pGSTN_0S129C$ and the 1,274-bp *Hpa*I-*Asp*718I fragment of pM307L into the 3,788-bp *Eag*I-*Asp*718I fragment of the vector $pSVN_0$. Thus, pSV/S129C/M307L encodes Pra with a Ser-to-Cys change at residue 129 and a Met-to-Leu change at residue 307. Plasmid pUC/S129C/M307L was constructed by replacement of a 1,628-bp *Eco*47III-*Asp*718I fragment of pRB4057 (26) with the same fragment derived from pSV/S129C/M307L.

Cleavage assay in *Escherichia coli*. Procedures for cleavage assays in the *E. coli* system were described previously (6, 32).

Isolation of the S129C protease-expressing cell lines. Vero cells were transformed with the plasmid pUC/S129C/M307L and pSVneo as described previously (13). G418-resistant colonies were grown into cultures and screened for the expression of the mutant protease and the ability to complement the growth of *A247S* virus. The cell clone BMS-S129C was used for experiments in this study (Fig. 1B).

Analysis of viral DNA and proteins. For slot blot analysis, DNA was prepared and analyzed essentially as described by Weinheimer et al. (53). Cells were infected with virus at a multiplicity of infection (MOI) of 10 PFU per cell unless otherwise specified.

For Western blot analysis, infected-cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13, 53). The procedure for Western blot analysis was described previously (13, 53). The anti-ICP35 monoclonal antibody (MAb) MCA406 (18) was used to detect ICP35 and protease-related products Pra, Prb, Na, and Nb (1:1,000 dilution; Serotec, Oxford, England).

Electron microscopy. The procedure for examination of infected cells by electron microscopy were was described previously (11).

***trans*-complementation assays.** The procedure for *trans* complementation has been described previously (13, 31). Vero cells were transfected with 3 μg of wt or mutant plasmids. At 20 h posttransfection, the cells were infected with 3 PFU of either *m*100 or *A247S* virus per cell and allowed to undergo a single cycle of infection. Virus yield was measured by plaque assay on BMS-MG22 and Vero cells.

Indirect immunofluorescence. Indirect immunofluorescence was performed was described previously (13). Primary antisera, specifically, anti-HCMV catalytic domain (1:50 dilution) or anti-HSV-1 N_0 (1:100 dilution), were detected by using fluorescein-conjugated goat anti-rabbit antibody (1:100 dilution).

RESULTS

Genetic evidence for distinct functions of the HSV-1 protease. The N-terminal 247-aa sequence of the HSV-1 protease (N_0) is enzymatically active but is insufficient to support viral growth (13, 30). This suggests that the C-terminal 388-aa sequence (Na) of the protease may perform a function required to support viral growth, perhaps by providing the specific signal required to localize N_0 to the site of capsid assembly (30). If localization is the only function of Na, then a chimeric protease consisting of the HCMV catalytic domain ($HCMVN_0$) and HSV-1 Na should retain functionality, since the CMV catalytic domain has the ability to cleave HSV substrates (55). To test this hypothesis, we constructed a plasmid containing a chimeric gene product consisting of the HCMV catalytic domain and HSV-1 Na (Fig. 1B) and examined expression and subcellular distribution of the gene product in transfected Vero cells by immunofluorescence. HCMV N_0, like HSV-1 N_0 (13), showed approximately equal intensities of cytoplasmic and nuclear staining (Fig. 2A). However, the chimeric gene product with the HCMV catalytic domain fused to HSV-1 Na, like HSV-1 wt Pra (13), demonstrated predominantly nuclear staining (Fig. 2B). These results suggest that when HSV-1 Na was fused to the HCMV catalytic domain, it restored the ability of the HCMV protease to localize in the nucleus.

We then examined whether the chimeric gene product retained the ability to autoprocess using the *E. coli* expression system. wt virus-infected Vero cells (Fig. 3A, lane 14) and the full-length wt HSV-1 protease (Pra) expressed in *E. coli* (Fig. 3A, lane 27) as well as the catalytic domain of the protease, N_0, coexpressed with ICP35 in *E. coli* (Fig. 3A, lanes 9 to 12) were used as positive controls. As expected, HSV-1 Na and Nb were produced when the chimeric protein was expressed alone or coexpressed with HSV-1 N_0 (Fig. 3A, lanes 15 to 18 and lanes 19 to 22). Bands between Prb and Na in these lanes may represent cleavage at the I site of the HCMV catalytic domain (2, 54). These results indicate that the chimeric protease has the ability to cleave itself at an R site composed of HCMV P and HSV-1 P′ residues. To determine whether the chimeric protease retains the ability to *trans* cleave ICP35, the full-length HSV-1 protease or the chimeric protease was coexpressed with ICP35 and examined by Western blotting (Fig. 3B). As expected, ICP35 c,d were *trans* cleaved into ICP35 e,f either by the HSV-1 protease (Fig. 3B, lanes 1 to 4) or by the chimeric protease (Fig. 3B, lanes 5 to 8).

To determine whether the HCMV-HSV chimeric protease could support the growth of the HSV-1 protease mutant viruses *m*100 and *A247S*, *trans*-complementation experiments were performed. The mutation in the *m*100 virus eliminates synthesis of the functional protease and, therefore, ICP35 cannot be cleaved (13). Although the mutation in *A247S* abolishes

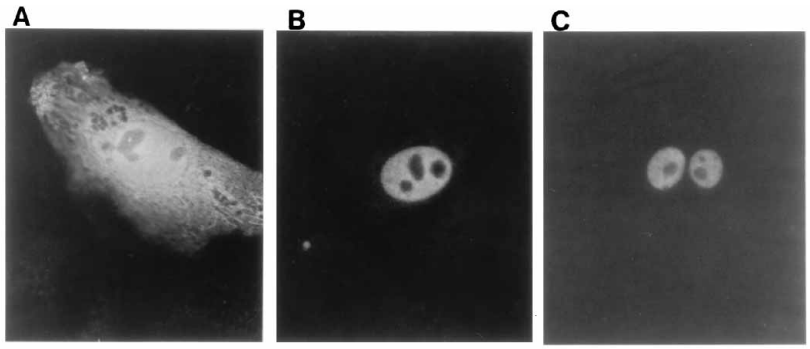


FIG. 2. Subcellular locations of mutant proteases. Vero cells were transfected with pSV/HCMVN_0 (A), pSV/HCMVN_0/HSVNa (B), or pSV/S129C (C). At 20 h posttransfection, cells were processed for an indirect immunofluorescence assay using polyclonal antisera specific for either HCMVN_0 (A and B) or HSV-1 N_0 (C).

the release of N_0, this mutant protease retains the ability to autoprocess at the M site and *trans* cleave ICP35 (30, 53). Neither of these mutant viruses grow on Vero cells but require HSV-1 protease-expressing BMS-MG22 cells for their propagation (13, 30). As shown in Table 1, transfection with the wt protease gene (Pra) complemented the growth of both mutants, while the HCMV-HSV chimeric protease (HCMVN_0/HSVNa) was not able to support the growth of these mutant viruses. Therefore, substitution of HSV-1 N_0 with the HCMV N_0 did not restore the complete functionality of the protease. These results suggest that HSV-1 N_0, in addition to its enzymatic activity, may have another function(s) required for viral replication.

Intragenic complementation of HSV-1 protease mutants. To further investigate the multiple functions of the HSV-1 protease, another protease mutant, S129C, was constructed. Plasmids pSV/S129C/M307L and pUC/S129C/M307L were derived from the plasmid pM307L (12), which expresses a protease with a Met-to-Leu change at residue 307 under the control of the simian virus 40 early promoter and its own promoter, respectively. The M307L mutation in the protease gene eliminates the synthesis of ICP35 but does not affect the protease functions required to support virus growth (13). Therefore, mutations in the protease gene should affect only the protease and not ICP35. The plasmid pSV/S129C/M307L encodes the mutant Pra with a Ser-to-Cys change at residue 129 and a Met-to-Leu change at residue 307 (Fig. 1B). Since the HSV-1 protease is a serine protease and the active site has been mapped to Ser-129 (11), neither the full-length S129C protease nor the mutant N_0 demonstrates enzymatic activity in the *E. coli* expression system (Fig. 3A, lanes 6 to 8 and lanes 3 to 5, respectively). However, the S129C protease is still a substrate for cleavage when coexpressed with wt N_0 (Fig. 3A, lanes 23 to 26). In addition, the mutant S129C protease, like wt Pra and the A247S protease (12, 13), localized to the nucleus (Fig. 2C). Therefore, we examined whether the defect of the noncleavable R site, but enzymatically active A247S protease could be complemented by providing the inactive, but cleavable mutant S129C protease in *trans.*

(i) Intragenic complementation between mutant virus *A247S* and mutant plasmid S129C. Complementation experiments were performed to determine whether expression of the S129C protease from a plasmid could complement the growth of *m*100 and *A247S* mutant viruses. Plasmids encoding wt pro-

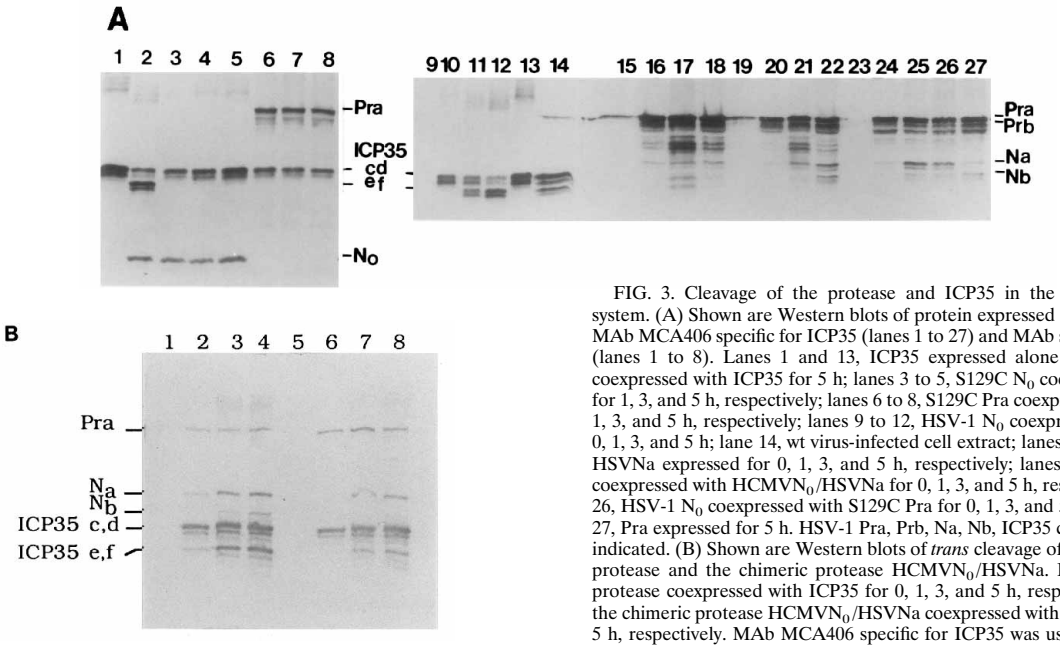


FIG. 3. Cleavage of the protease and ICP35 in the *E. coli* coexpression system. (A) Shown are Western blots of protein expressed in *E. coli* probed with MAb MCA406 specific for ICP35 (lanes 1 to 27) and MAb specific for HSV-1 N_0 (lanes 1 to 8). Lanes 1 and 13, ICP35 expressed alone for 5 h; lane 2, N_0 coexpressed with ICP35 for 5 h; lanes 3 to 5, S129C N_0 coexpressed with ICP35 for 1, 3, and 5 h, respectively; lanes 6 to 8, S129C Pra coexpressed with ICP35 for 1, 3, and 5 h, respectively; lanes 9 to 12, HSV-1 N_0 coexpressed with ICP35 for 0, 1, 3, and 5 h; lane 14, wt virus-infected cell extract; lanes 15 to 18, HCMVN_0/HSVNa expressed for 0, 1, 3, and 5 h, respectively; lanes 19 to 22, HSV-1 N_0 coexpressed with HCMVN_0/HSVNa for 0, 1, 3, and 5 h, respectively; lanes 23 to 26, HSV-1 N_0 coexpressed with S129C Pra for 0, 1, 3, and 5 h, respectively; lane 27, Pra expressed for 5 h. HSV-1 Pra, Prb, Na, Nb, ICP35 c,d and e,f and N_0 are indicated. (B) Shown are Western blots of *trans* cleavage of ICP35 by the HSV-1 protease and the chimeric protease HCMVN_0/HSVNa. Lanes 1 to 4, HSV-1 protease coexpressed with ICP35 for 0, 1, 3, and 5 h, respectively; lanes 5 to 8, the chimeric protease HCMVN_0/HSVNa coexpressed with ICP35 for 0, 1, 3, and 5 h, respectively. MAb MCA406 specific for ICP35 was used.

TABLE 1. Intragenic complementation between HSV-1 protease mutants[a]

Gene transfected	Virus superinfected	Titer (PFU/ml)[b]		Complementation index[c]	
		Expt 1	Expt 2	Expt 1	Expt 2
pUC18	*m*100	2.4×10^3	5.2×10^2	1	1
	A247S	1.0×10^2	1.8×10^2	1	1
Pra	*m*100	5.4×10^5	1.4×10^5	225	265
	A247S	6.0×10^4	2.4×10^4	600	133
S129C/M307L	*m*100	2.4×10^3	7.6×10^2	1	1.5
	A247S	8.0×10^4	5.4×10^4	800	300
$HCMVN_0$/HSVNa	*m*100	3.0×10^3	7.8×10^2	1.3	1.5
	A247S	2.8×10^2	8.0×10^2	2.8	4.4

[a] Vero cells were transfected with the plasmids indicated. At 20 h posttransfection, cells were infected with either *m*100 or *A247S* at 3 PFU per cell and incubated for an additional 20 h before being harvested.

[b] Viral yield was determined by plaque assays on BMS-MG22 cells at 2 days p.i.

[c] Expressed as viral yield relative to that for cells transfected with pUC18 DNA.

tease Pra and $HCMVN_0$/HSVNa were also used in these experiments. Neither the S129C/M307L protease nor the chimeric protease was able to complement the growth of *m*100 (Table 1). However, the S129C/M307L protease, but not the $HCMVN_0$/HSVNa protease, could fully complement the growth of *A247S* virus (Table 1). These results suggest that the growth of *A247S* virus was complemented by a function provided by the N_0 domain of the S129C protease.

(ii) Phenotype of the mutant S129C. To determine the basis of intragenic complementation between HSV-1 protease mutants, we isolated a cell line, named BMS-S129C, which expresses the mutant S129C/M307L protease. The enzymatic activity of the mutant protease from infected-cell extracts was examined. In these assays, ICP35 was provided by mutant viruses, either *m*100 or *A247S*. Cell extracts were prepared at 10 h postinfection (p.i.), separated by SDS-PAGE, and analyzed by Western blotting (Fig. 4). Since the amino acid sequence of ICP35 is identical to the C-terminal portion of the protease (26, 33), the MAb we used, MCA406, reacted with ICP35 and several of the autoproteolytic products of the protease. In wt virus-infected cells, ICP35 c,d was processed to ICP35 e,f (Fig. 4, lanes 1, 2, and 3). However, in *m*100-infected BMS-S129C cells (Fig. 4, lane 6), like *m*100-infected Vero cells (Fig. 4, lane 4), normal amounts of ICP35 c,d were produced but not processed to the ICP35 e,f forms. This result again demonstrates that the mutant S129C protease does not have the ability to cleave ICP35. As we reported recently (30), the mutant A247S protease retains enzymatic activity, but cleavage of ICP35 c,d to ICP35 e,f is less than in wt virus-infected Vero cells (Fig. 4, compare lanes 1 and 7). This enzymatic activity is restored to wt level in *A247S*-infected BMS-S129C cells, as evidenced by the increased processing of ICP35 (Fig. 4, compare lanes 1, 7, and 9).

(iii) Evidence that the proteolytic processing events relevant to capsid assembly occur inside capsids. Although fully processed protease (N_0 and Nb) and ICP35 (ICP35 e,f) are present in B capsids (15, 37, 52, 53), it is unknown whether it is necessary for the cleavages to take place inside capsids in order to form infectious particles. The intragenic complementation between our two mutants may provide a tool to address this question. We postulated that if the cleavages occur prior to capsid assembly, one would expect that only fully processed protease (N_0 and Nb derived from S129C protease) and ICP35 e,f derived from *A247S* virus-infected cells would be present in B capsids; if the cleavages occur during or after capsid assembly, one would expect that not only fully processed protease but also the partially processed protease Prb, derived from *A247S* virus-infected cells could be detected in B capsids. We, therefore, examined whether the enzymatically active, but noncleavable R-site A247S protease is present in capsids isolated from *A247S*-infected BMS-S129C cells.

wt or *A247S* virus-infected BMS-S129C cell extracts were subjected to centrifugation through 20 to 50% sucrose gradients. Extracts from *m*100-infected BMS-S129C cells and *A247S*-infected Vero cells were used as negative controls. After sedimentation of infected-cell lysates, capsid bands were visualized only in extracts of wt virus- and *A247S*-infected BMS-S129C cells, but not in extracts of *m*100-infected BMS-S129C cells or *A247S*-infected Vero cells (results not shown). Fractions were collected, subjected to SDS-PAGE, and analyzed by Western blotting with MAb MCA406 (Fig. 5). As expected, in wt virus-infected BMS-S129C cell extracts, Nb and ICP35 e,f were observed in fractions 10 to 12 corresponding to B capsids (Fig. 5A). In contrast, no apparent Nb and ICP35 peak was present in similar fractions from the *m*100-infected BMS-S129C cell extracts (Fig. 5B) or *A247S*-infected Vero cells (results not shown). Most of the unprocessed ICP35 c,d appeared to accumulate in the first few fractions (the top) of the gradient in *m*100-infected BMS-S129C cell extracts (Fig. 5B). In mutant *A247S*-infected BMS-S129C cells, Prb, apparently derived from the A247S protease (Pra), was observed in addition to Nb and ICP35 e,f in the fractions where wt B capsids sediment (Fig. 5C). To ensure that bands in samples of fractions 10 to 12 in Fig. 5C were Prb and Nb, not Pra and ICP35 c,d, we reexamined fractions 10 and 11 shown in Fig. 5A and C with controls side by side. Both Pra and Prb were present in the control sample (Fig. 5D, lane 1), and as expected, neither Pra nor Prb was present in these B-capsid fractions of wt virus-infected BMS-S129C cells (Fig. 5D, lanes 4 and 5). In contrast, Prb is present in these fractions of *A247S*-infected BMS-S129C cells (Fig. 5D, lanes 2 and 3). When the same samples from fractions 10 and 11 of Fig. 5A and C were examined with a polyclonal anti-N_0 serum, the catalytic domain of the HSV-1 protease (N_0) was detected in wt- and *A247S* virus-infected BMS-S129C cell extracts (Fig. 5D, lanes 6 to 10). In order to generate N_0, the full-length protease (wt Pra or S129C Pra) must be cleaved. Therefore, bands in fractions 10 and 11 of Fig. 5A and C must be Nb, not ICP35 c,d. This conclusion was further confirmed by the fact that these bands did not react with an antiserum specific for the

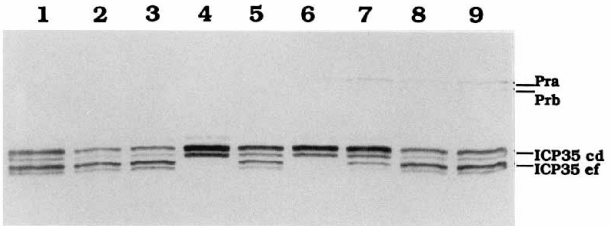
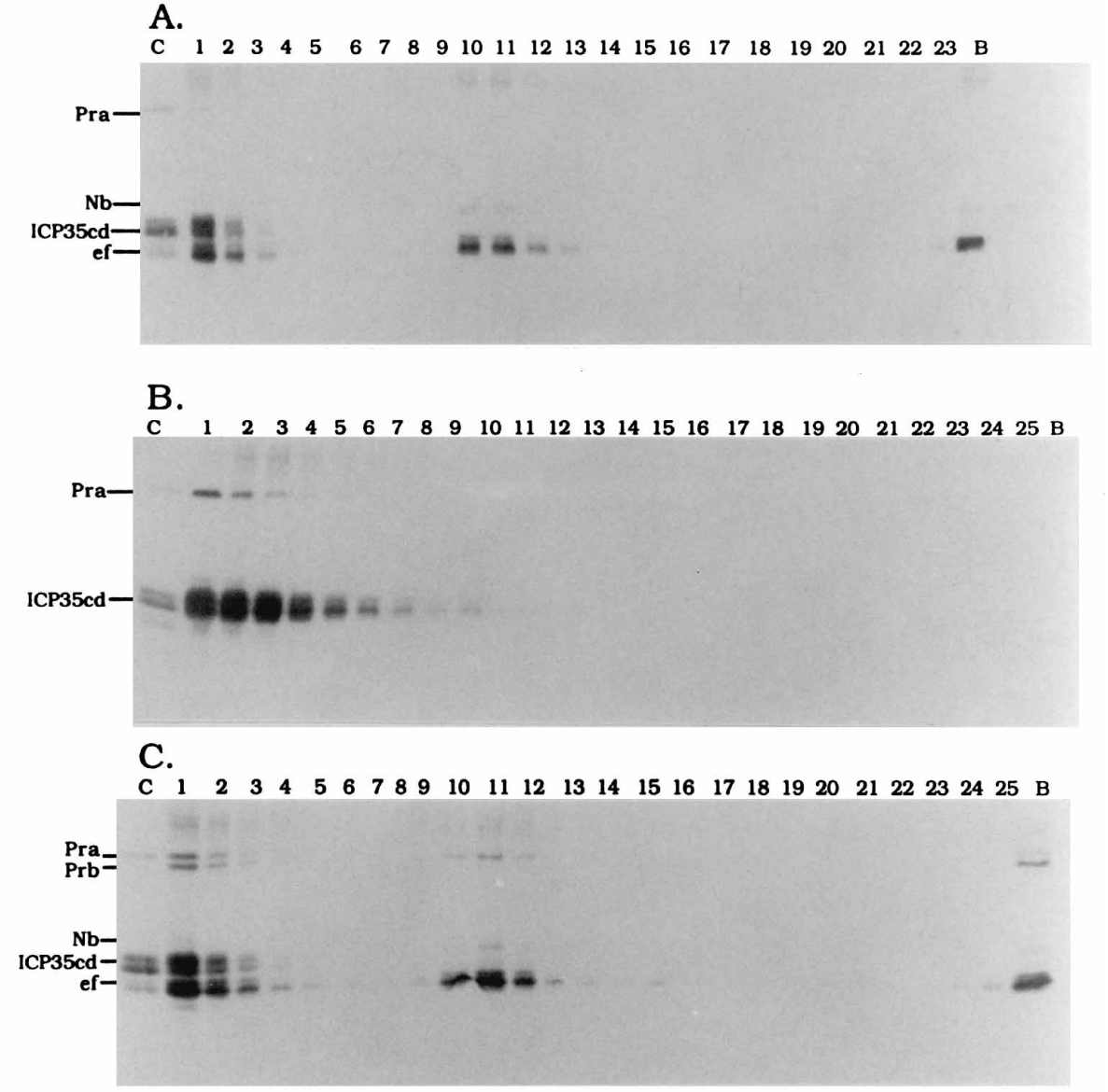


FIG. 4. Western blot analysis of HSV-1 protease-related proteins in infected cells. Vero (lanes 1, 4, and 7), BMS-MG22 (lanes 2, 5, and 8), and BMS-S129C (lanes 3, 6, and 9) cells were infected with wt (lanes 1 to 3), *m*100 (lanes 4 to 6), or *A247S* (lanes 7 to 9) virus. Total proteins were prepared at 10 h p.i., separated by SDS-PAGE, and transferred to a nitrocellulose filter. The filter was probed with the MAb MCA406.



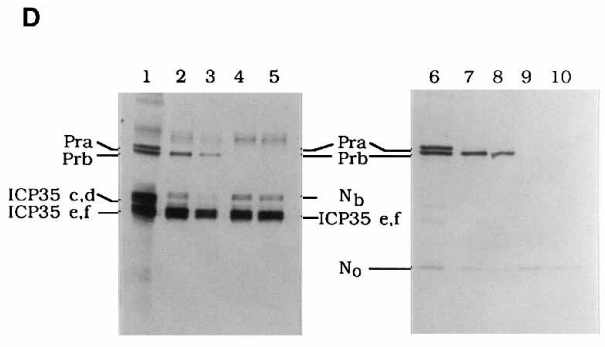


FIG. 5. Sucrose gradient analysis of infected-cell lysates. BMS-S129C cells were infected with either wt (A), *m*100 (B), or *A247S* (C) virus at an MOI of 10 PFU per cell for 16 h. Total extracts were prepared and layered onto 20 to 50% sucrose gradients and centrifuged at 77,175 × *g* for 1 h. Fractions were collected, and proteins were subjected to SDS-PAGE (lanes 1 to 23 or 25). Proteins were transferred to a nitrocellulose filter probed with MAb MCA406. The sedimentation direction is from left (top) to right (bottom). Lanes C, wt virus-infected cell extract used as a control; lanes B, pellets of gradients. (D) Samples from fractions 10 and 11 of wt virus-infected BMS-S129C cell extracts shown in Fig. 5A and *A247S*-infected BMS-S129C cell extracts shown in Fig. 5C were examined by Western blotting. Lanes 2 and 7 contain samples from fraction 10, and lanes 3 and 8 contain samples from fraction 11 of Fig. 5C. Lanes 4 and 9 contain samples from fraction 10, and lanes 5 and 10 contain samples from fraction 11 of Fig. 5A. Lanes 1 and 6 containing samples derived from fraction 1 of *A247S*-infected BMS-S129C cell extracts were used as Pra, Prb, ICP35 c,d and ICP35 e,f controls. MAb MCA406 was used for lanes 1 to 5, and a polyclonal anti-N_0 serum was used for lanes 6 to 10.

C-terminal 25-aa peptide (results not shown). In addition, Prb, not Pra, was also detected by this anti-N_0 serum only in fractions 10 and 11 of *A247S*-infected S129C cells, not in those of wt virus-infected cells (Fig. 5D, compare lanes 7 and 8 with lanes 9 and 10). Taken together, these results demonstrate that the precursor protease can be incorporated into capsids. This is consistent with the hypothesis that the capsids are the relevant site of proteolytic processing during the formation of infectious virus (30).

(iv) Encapsidation of viral DNA. We then examined whether

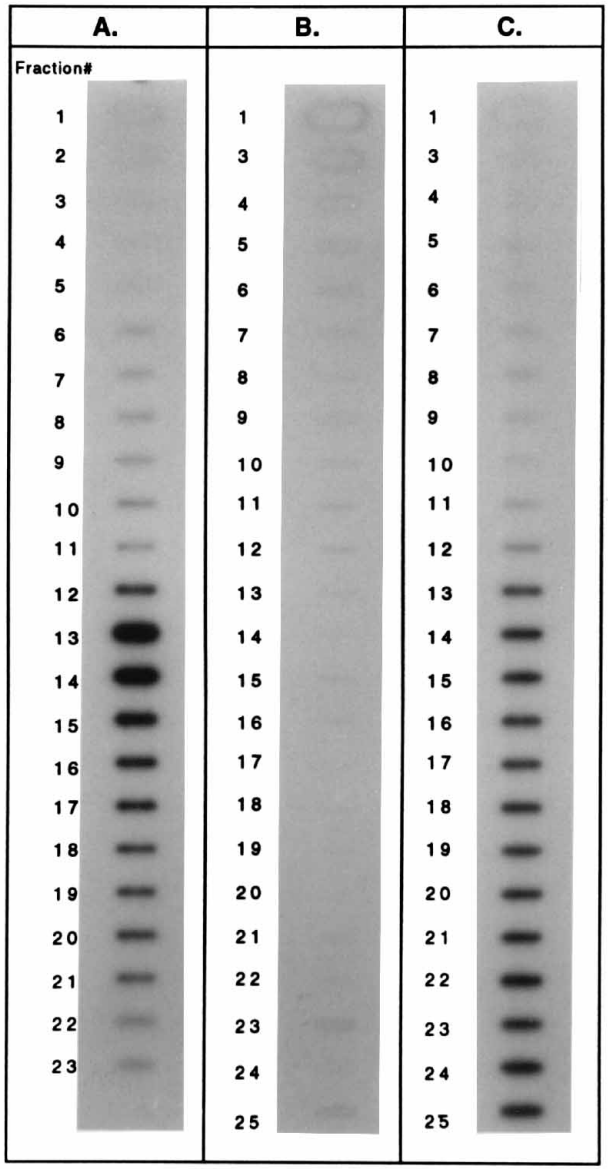


FIG. 6. Encapsidation of viral DNA. Aliquots from each fraction of the sucrose gradients shown in Fig. 5 were prepared and hybridized to a ^{32}P-labeled plasmid, pUCICP35. BMS-S129C cells infected with wt virus (A), *m*100 (B), and *A247S* (C).

viral DNA is packaged in the capsids formed in *A247S*-infected BMS-S129C cells. Aliquots from each fraction of the sucrose gradients shown in Fig. 5 were prepared and hybridized to a ^{32}P-labeled plasmid, pUCICP35. As shown in Fig. 6A, the B-capsid fractions containing Nb and ICP35 e,f did not contain significant amounts of viral DNA (fractions 10 to 12); the wt viral DNA localized in faster sedimenting fractions, peaking at fractions 13 and 14. Similarly, the mutant viral DNA also localized in faster sedimenting fractions but appeared to sediment from the C-capsid fractions (fraction 13) to the bottom of the gradient of the *A247S*-infected BMS-S129C cell extracts (compare Fig. 6A and C). The nature of this observation is unknown at this time. No significant amount of viral DNA was detected throughout the gradient of *m*100-infected BMS-S129C cell extract (Fig. 6B). We, therefore, conclude that the intragenetic complementation between the two mutant proteases restored the wt protease functions required for encapsidation of viral DNA.

(v) Electron microscopic study. Thin sections of virus-infected cells were examined to determine whether capsid structures are formed. As expected, cells infected with wt virus were found to contain three types of capsids, A, B, and C capsids, as well as virions (Fig. 7A and results not shown). Although capsid structures were observed in *A247S*-infected Vero cells (Fig. 7C) and *m*100-infected BMS-S129C cells (Fig. 7D), all were the electron-transparent (DNA$^-$) capsids. In contrast, in *A247S*-infected BMS-S129C cells, three types of wt-like capsids as well as virions were observed (Fig. 7B and E and results not shown). The size of B capsids in *A247S*-infected BMS-S129C cells was very similar to that of wt virus-infected BMS-S129C cells. However, significant numbers of capsids, especially in the nuclear section (Fig. 7E), were aberrant.

(vi) Growth of *A247S* in BMS-S129C cells. To determine whether *A247S* grows on BMS-S129C cells, plaque assays were performed. The mutant *A247S* virus formed slightly smaller plaques than wt virus on BMS-S129C cells (results not shown). Since the plaques were smaller, we performed a single-cycle growth experiment to examine the growth property of *A247S* virus on BMS-S129C cells. The yield of wt virus on BMS-S129C cells was comparable to those on Vero cells at MOIs of 5 and 0.1, indicating that the mutant S129C protease did not exhibit a negative *trans*-dominant phenotype (Table 2). In agreement with *trans*-complementation experiments, the *m*100 virus did not grow on either Vero or BMS-S129C cells. As we reported recently, the *A247S* virus exhibits a slightly *trans*-dominant phenotype (30), and the yield of this mutant on the protease-expressing BMS-MG22 cell line was approximately three- to fivefold lower at MOIs of 5 and 0.1 than that of *m*100 virus. Although *A247S* virus failed to grow on Vero cells, the yield of *A247S* virus on BMS-S129C cells was close to that of the mutant on BMS-MG22 cells at an MOI of 5, but the yields were sevenfold lower at an MOI of 0.1. Since recombination does occur at very low frequency between homologous DNA sequences present in the mutant genomes and in the chromosomes of the transformed cell lines, the samples from *A247S*-infected cells were examined for wt recombination. Table 2 shows that wt recombination in *A247S* virus-infected BMS-MG22 and BMS-S129C cells was almost undetectable at the dilutions we examined. Because BMS-S129C cells support the growth of only *A247S*, and not of *m*100, we conclude that the complementation between two mutant proteases reconstitutes the wt protease activity required to support viral growth.

DISCUSSION

In this study, two mutant proteases were used to examine functional domains of the HSV-1 protease. Neither of these mutant proteases was able to support the growth of the protease deletion mutant virus. One mutant protease is expressed from the virus *A247S*, which contains an Ala-to-Ser change at residue 247. This mutant protease fails to autoprocess at the R site but retains the ability to cleave at the M site and also the ability to cleave its substrate ICP35 (30). The other mutant protease, expressed from a transformed cell line, contains a Ser-to-Cys mutation at its active site, Ser-129. The mutant S129C protease, unlike the A247S protease, lacks enzymatic activity but is a substrate for an active protease. When these two mutant proteases are provided in *trans*, they are able to restore viral growth, suggesting that an essential function is being supplied by the S129C protease. The possibility that the Nb domain of the S129C protease was supplying the essential function was ruled out, since the $HCMVN_0$/HSVNa chimera

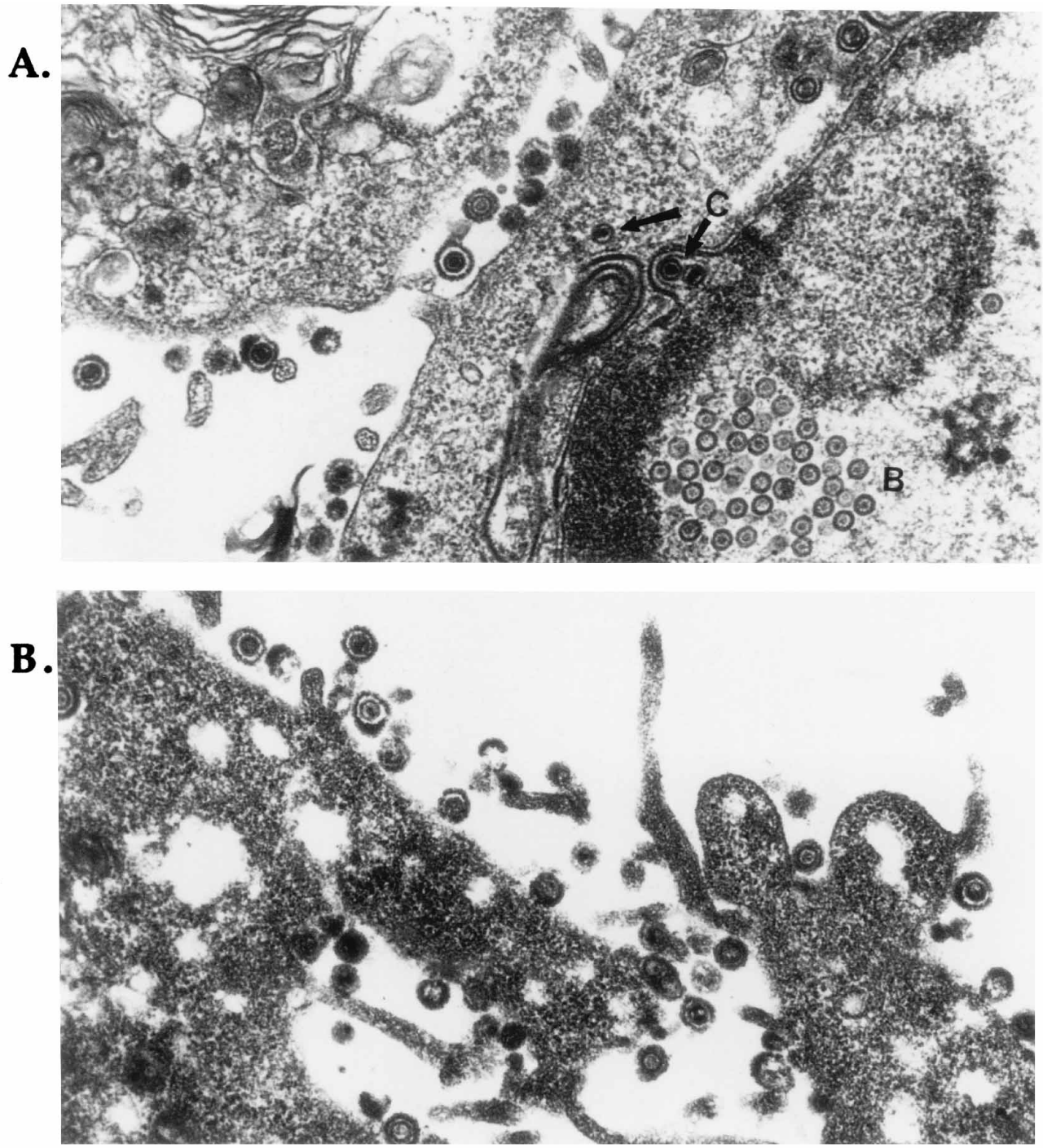


FIG. 7. Electron micrographs of thin sections of virus-infected cells. Cells were infected with different viruses at an MOI of 10 PFU per cell. Cells were fixed and prepared at 16 h p.i. as described in Materials and Methods. (A) wt virus-infected BMS-S129C cells; (B) *A247S*-infected BMS-S129C cells; (C) *A247S*-infected Vero cells; (D) *m*100-infected BMS-S129C cells; (E) *A247S*-infected BMS-S129C cells. Magnifications, ×32,500 (for panels A and B) and ×107,250 (for panels C, D, and E).

did not support the growth of either the *m*100 or *A247S* virus. Our results suggest that HSV-1 protease is composed of multiple functional domains and some of these domains can be genetically separated.

The catalytic domain (N_0) and Nb of the protease as well as ICP35 e,f are all components of B capsids (15, 37, 52, 53). Intrageneic complementation between two mutant proteases suggests that N_0 may have another function(s) besides enzymatic activity required for viral growth. There are several possible roles that N_0 may play inside B capsids. First, following its release from Pra inside capsids, N_0 may be responsible for *trans* cleaving ICP35. In this case, *trans* processing of ICP35 by N_0 must be an extremely fast event, or the capsids containing unprocessed ICP35 are unstable, since capsids containing N_0

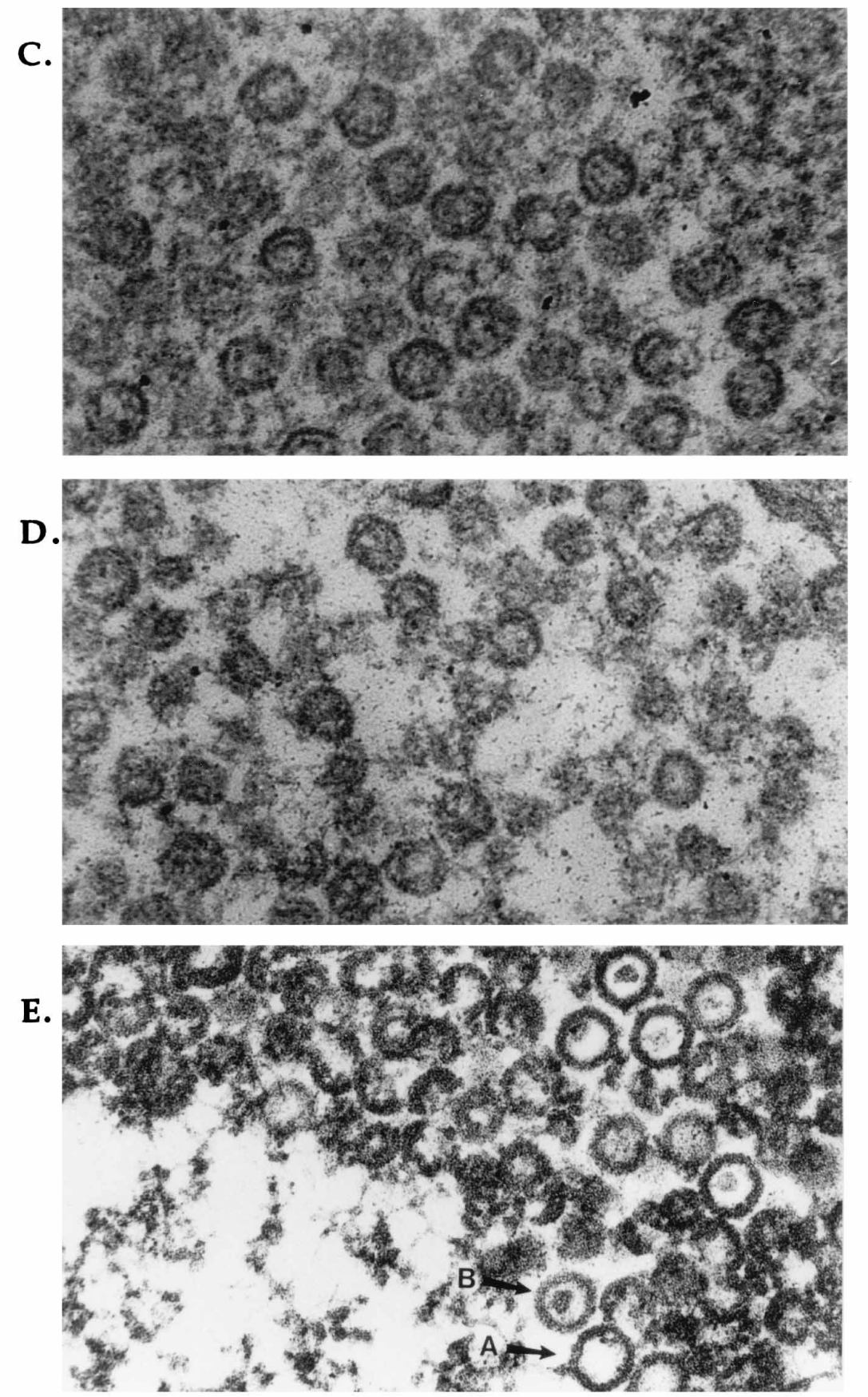


FIG. 7—*Continued.*

TABLE 2. Single-cycle growth of wt and mutant viruses on different cell lines[a]

Virus and cell types	Yield (PFU/cell)			
	BMS-MG22 cells		Vero cells	
	MOI = 0.1	MOI = 5	MOI = 0.1	MOI = 5
wt (KOS1.1)				
Vero	83	196	77	167
BMS-MG22	71	191	42	173
BMS-S129C	101	244	131	226
*m*100				
Vero	0.0019	0.038	<0.00006	0.00012
BMS-MG22	42	131	<0.006	0.006
BMS-S129C	0.0026	0.13	0.00012	0.00024
A247S				
Vero	0.0013	0.0042	<0.0006	<0.0006
BMS-MG22	8.3	48	<0.0006	0.0012
BMS-S129C	1.1	26	<0.0006	0.0012

[a] Cells were infected with viruses at the indicated MOIs, incubated at 37°C for 18 h, and harvested. Titers of progeny viruses were determined in BMS-MG22 and Vero cells.

and unprocessed ICP35 have not been observed. A second possible role for N_0, like the bacteriophage T4 prohead protease (T4PPase), may be to digest itself, Nb, and ICP35 e,f during the encapsidation of viral DNA (17, 48). In this case, either the specificity of the protease must be changed or the specificity of the protease is dependent on the substrate conformation rather than its primary sequence, as a consensus cleavage recognition site has not been identified elsewhere in these proteins. Third, N_0, like ICP35 e,f, may play a structural role in the formation of B capsids. This hypothesis is consistent with several observations: (i) the HCMV catalytic domain can provide enzymatic activity but cannot support viral growth; (ii) processing of ICP35 in *A247S*-infected Vero cells is insufficient to support viral growth (29), suggesting that the protease has a function which is distinct from its enzymatic activity; and (iii) N_0 is a component of B capsids. Although capsid structures were observed from HSV-1 protease mutant-infected cells by electron microscopy, such capsids have not been isolated by sucrose gradient centrifugation (13, 29, 44), suggesting that release of N_0 is required for capsid stability. The exact role of N_0, whether enzymatic, structural or undefined, has yet to be determined.

HSV-1 protease undergoes autoprocessing at the M and R sites, releasing N_0, Nb, and a 25-aa peptide; however, whether this autoprocessing occurs in *cis* or in *trans* is unknown. Although our intragenic complementation experiments did not exclude the possibility of *cis* cleavage, it clearly demonstrated that *trans* cleavage of the mutant protease S129C by the A247S protease can lead to functional capsid assembly and produce infectious virus.

Since processing of ICP35 is restored to wt level in *A247S*-infected BMS-S129C cells, we carefully examined the S129C protease for residual enzymatic activity. The inability of the S129C protease to *trans* cleave ICP35 in the *E. coli* coexpression system or in *m*100 virus-infected BMS-S129C cells suggests that the mutant protease lacks enzymatic activity. Our experiments also excluded the possibility that the elevated processing of ICP35 in *A247S*-infected BMS-S129C cells is due to the restoration of enzymatic activity following the release of S129C N_0 from full-length S129C protease, since S129C N_0 is not proteolytically active. The restored processing of ICP35 observed in *A247S*-infected BMS-S129C cells is more than additive in Fig. 4 and is further evidence that the restored cleavage of ICP35 is not due to residual enzymatic activity of the mutant S129C protease. The most convincing evidence supporting the conclusion that the S129C protease is inactive is derived from single-cycle growth experiments. Failure of S129C protease to support the growth of *m*100 clearly demonstrated that at least one essential function of the mutant S129C protease is inactivated.

A key question regarding the HSV-1 virion maturation pathway remains. At what point during capsid assembly does the autocleavage of the protease and *trans* processing of ICP35 occur? During T4 phage head assembly, gp21 is incorporated into the prohead core as an inactive zymogen which is activated only after the assembly of the shell (48), and no cleavage takes place in mutants in which assembly is blocked. In contrast, the enzymatic activity of the HSV-1 protease is not linked to the assembly of capsids, since the protease can undergo autoprocessing and *trans* cleave ICP35 in transfected cells (26–28), an *E. coli* coexpression system (6, 32), the baculovirus system (49, 51), and assembly-negative mutant virus-infected Vero cells (8). In the latter case, no capsid structures were observed by either sucrose gradient analysis or electron microscopy (7). However, these cleavage events may not reflect the exact role of the protease and ICP35 during the virion maturation. Temperature shift experiments with HSV-1 protease mutant *ts*1201 virus-infected cells indicated that cleavages may occur inside of the capsids, and only these capsids can mature to package viral DNA (44). However, these results cannot rule out the possibility that aberrant capsids may be dissociated and reassembled or that free, preexisting capsid proteins were assembled into normal capsids when the temperature was shifted from nonpermissive to permissive. On the basis of the baculovirus system, Thomsen et al. (50) also proposed a model where the uncleaved protease, Pra, and its substrate ICP35 are used to assemble B capsids. An interesting feature of the intragenic complementation between the two mutant proteases from our experiments is that Prb as well as Nb and N_0 is present in the B capsids of *A247S*-infected BMS-S129C cells. These capsids retain the ability to package viral DNA, as demonstrated by the presence of viral DNA in gradient fractions corresponding to C capsids, and mature into infectious virus. In *m*100-infected BMS-S129C cells and in *A247S*-infected Vero cells, B capsids could not be isolated and viral DNA was not encapsidated. These results strongly suggest that Prb, N_0, Nb, and ICP35 e,f must coexist in the same B capsids in *A247S*-infected BMS-S129C cells. Our results and the results of others (6, 7, 10, 27–29) support the hypothesis that when the protease and its substrate ICP35 interact, the specific cleavage occurs; however, only those cleavages that occur during or after capsid assembly are able to form functional capsids capable of packaging of viral DNA.

It was surprising to observe that the processing of ICP35 in *A247S*-infected BMS-S129C cells was restored to wt levels. It has been recently reported that in an in vitro assay, HSV-1 protease activity is stimulated over 100-fold by water structure-forming cosolvents, such as antichaotrophic salts (18, 57). This stimulation effect is most likely due to changes in the conformation of the substrate as well as the protease, since both the susceptibility of the substrate to proteolysis by trypsin and the protein fluorescence spectra of the protease are altered in the presence of solvents (57). These cosolvents may be mimicking the conformation of the protease under optimum conditions, i.e., inside the capsid. It is therefore conceivable that the restored processing of ICP35 in *A247S*-infected BMS-S129C

cells is completely due to the conditions of the capsid environment.

The formation of heterodimers and multimers of two genetically discrete mutant protein molecules provides a common mechanism for intragenic complementation (47, 58). While this article was being revised, Darke et al. (5) reported that the HCMV protease forms a dimer. A simple model for our intragenic complementation results is that the A247S and S129C proteases form a functionally active heterodimer. The full-length A247S protease provides the enzymatic activity in order to *trans* cleave ICP35 and release N_0 from the S129C protease. The N_0 domain of S129C protease then supplies another essential function for the formation of functional capsids which can mature into infectious virus. Further experiments will directly test whether these two mutant proteases form a heterodimer during or after capsid assembly.

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